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13. ABSTRACT (Maximum 200 Words)

We hypothesize that a simple, interpretable pattern can be derived by following changes in breast cells from a single individual as they undergo progression from normal appearing ductular forms to the appearance of carcinoma-in-situ and invasive cancer. We proposed to 1) Collect enough microdissected tissue representative of each of the morphologically different stages of early breast cancer progression in the MCF10AT model to obtain RNA for microarray analysis of gene expression and PCR-amplification of DNA for analysis of global and gene specific CpG island methylation. 2) Compare the methylation patterns of candidate genes in these tissues with those of MCF10A, MCF10AT and MCF10DCIS.com. cells in culture. 3) Prepare a CpG island methylation profile and a gene expression profile for each of the tissue types and cell lines.

This year we completed examination of DNA methylation in 15 CpG islands in genes whose silencing by methylation has been linked to cancer development using MCF-7 and MCF10A cells and 6 cell lines in the MCF10AT model.. The results of this analysis have been coupled with gene expression data and validate our methods. We have also collected sufficient tumor tissue from xenografts to carry out analysis of methylation status in xenografts during progression.

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I. INTRODUCTION:

There is a growing literature that suggests a causal role for aberrant DNA methylation in breast cancer development and a number of breast cancer cell lines have been shown to carry genes silenced by methylation. Genes that are up- or down- regulated in human breast cancers are already being studied by microarray gene expression analysis, and several studies of changes in methylation at CpG islands in cultured breast cells and breast tumor samples have been reported. However, even though classification of tumors on the basis of gene expression patterns and methylation profiles are being derived from candidate genes in a variety of tumors, the genetic, epigenetic, cellular and morphological heterogeneity of the breast tumor samples derived from different patients greatly complicates derivation of mechanistic explanations of tumor development from patient material. Thus, it would be of great utility to be able to follow the changes in breast tissue from single individuals as breast cancer develops and progresses.

Our objective in these studies is to develop a profile of epigenetic and gene expression changes occurring during the early stages of breast cancer progression. Our hypothesis is that a simpler, interpretable pattern of changes, similar to that developed for progression of colon cancer in individuals with familial polyposis coli can be derived by studying the changes that occur in breast cells from a single individual as they undergo change from normal appearing ductular forms, through hyperplasia to the appearance of carcinoma-in-situ and locally invasive cancer. The aims are to: 1) Collect enough microdissected tissue representative of each of the morphologically different stages of early breast cancer progression in the MCF10AT model to allow preparation of RNA for microarray analysis of gene expression and PCR-amplification of DNA for analysis of global and gene specific CpG island methylation. 2) To compare the methylation patterns of candidate genes in these tissues with those of MCF10A, MCF10AT and MCF10DCIS.com., cells grown in culture. 3) To prepare a methylated CpG island profile for and a gene expression profile for each of the tissue types and cell lines.

II. BODY:

Task 1. Optimize techniques for analysis of gene array and CpG island analysis using cultured MCF10AT cells. (Months 1-12)

a. Determine minimum number of cells needed to accurately detect differential expression of candidate genes by microarray.
b. Determine minimum number of cells required to accurately detect differences in CpG islands by array.
c. Sequence CpG islands that demonstrate differential cross-hybridization with RT-PCR products from cultured MCF10A and MCF10AT and MCF10DCIS.com cells.

Task 1 a and b are completed. We have determined that ~ 2 μg of DNA(~2 x 10^6 cells) is required to provide sufficient probe for one array. This is well within the number of cells we can obtain from our tissue culture experiments. However, it is unlikely that currently available laser capture methods can be used to obtain this number of cells on a routine basis. We now

have access to the new Leica laser capture microscope, which will help increase our cell yield ~10 fold to 20,000-30,000 cells. This is still at least 2 logs less than needed. Thus, we will extend this task to develop methods for increasing the degree of amplification of the CpG island DNA and enhancing the levels of production and label density of the probes without altering the relative contributions of different sizes of CpG island fragments.

In contrast to the limitations of cell numbers needed for CpG island microarray analysis, it is relatively easy to obtain differential expression data with RNA from 1,500 cells, well within the limits of our ability for capturing cells by laser capture microdissection.. Our initial studies also indicate that we can easily carry out quantitative methylation specific PCR on DNA recovered from ~50 cells obtained by laser capture microdissection. Thus, our strategy in the coming year will be to identify potentially important differentially methylated CpG islands (Task 1 c) on our 40,000 CpG island clone arrays using PCR products from cultured cells. We will then concentrate on the use of methylation specific PCR of bisulfite modified DNA to examine the methylation of these CpG islands in morphologically different regions of microdissected MCF10 AT tumors.

Task 2. Collect sufficient xenograft tissue to begin analysis of progression in xenografts. (Months 2-15)

a. Quantitate and optimize recovery of cells from defined areas of lesions.
b. Quantitate recovery of DNA and RNA from microdissected tissues
c. Optimize RT-PCR and CpG island PCR from microdissected tissue.
d. Inject 50 N/B mice in each flank with MCF10AT cells and another 50 with MCF10DCIS.com cells. Collect lesions at times indicated in methods

Task 2. a and b are completed (See above). We have collected tumors formed by MCF10DCIS.com [2] for our initial analysis. We have also collected tumors formed by several highly malignant breast tumor lines derived from MCF10AT cells by Dr. Fred Miller at Karmanos Inst. (MCF10CA1a, MCF10CA1d, MCF10CA1h) [3]. Since all of these lines except MCF10AT form large tumors in 4-6 weeks, we have collected sufficient tissue to initiate microdissection and obtain DNA and RNA for analysis. We have also collected lungs from mice bearing CA1h tumors, since Dr. Miller has shown that cells from these tumors metastasize to the lung. Unfortunately, we had two problems with obtaining lesions from MCF10AT itself. These lesions require triple immune deficient mice for optimal growth. In our first year of study, only 30 female mice could be obtained from Taconic Farms. I did not think it was prudent to set up a schedule of regular deliveries of mice specifically bred for us as required by the supplier until we had done some preliminary experiments to determine how much MCF10AT tissue we could obtain. This meant we had to settle for "excess progeny" not needed by investigators with contracts. In our first experiment, no lesions were formed. We got fresh cultures from Dr. Miller who has now been able to maintain the line in ordinary "nude" mice. We are now ready to harvest the first lesions from these cells and expect to progress well with Task 1d in the coming year.
As noted above, we have already optimized methylation specific Q-PCR and RT-PCR from microdissected tumor tissues other than MCF10AT.

Task 3. Complete analysis of methylation and expression of candidate genes in cultured MCF10A; MCF10AT and MCF10DCIS.com (Months 1-12).

We have made excellent progress on this aim. Our first studies have focused on determining the extent of methylation and expression of a set of genes known to be hypermethylated in cancer using cultured cells of the MCF10 model system for breast cancer progression. Since we intend to carry out extensive studies on both cultured cells and tumors using CpG island microarray, we picked a limited set of genes to allow comparison of the results obtained by CpG island microarray with those obtained by methylation specific PCR and those obtained by the "gold standard" bisulfite sequencing. This is important to do because CpG island microarray by nature will only detect loss or gain of methylation at sites recognized by restriction endonucleases whose cleavage is blocked by methylation at CpG sites (BstUI, HpaII, etc). Methylation specific PCR allows a rapid check on methylation status for known CpG islands but is more limited than the CpG island microarray in terms of the size of the area that can be examined. The results of this part of the study have been important not only from the aspect of obtaining new information about methylation of these genes in cell lines representative of different stages in breast cancer progression but in interpreting data obtained by CpG island microarray.

1. *CpG Island microarray analysis:* We have made "mini-microarrays" of CpG islands of 16 tumor suppressor genes known to be hypermethylated in cancer (APC, p14 ARF, E-cadherin, p16, p15, estrogen receptor, glutathione s-transferase P1, HIC1, MGMT, MLH1, RB, RIZ1, BRCA1, TMS1, TIMP3 and NOEY2 as well as controls (cloned mitochondrial and ribosomal DNA and MYOD1). In each case, the cloned fragment was from a region flanked by MseI sites. This is the same restriction enzyme site used for cloning CpG islands during preparation of the CpG island library [4]. A typical mini-microarray using MCF10AT cell DNA as probe is shown in Fig. 1. A "yellow" spot indicates that intact CpG island DNA was present before and after cutting the genomic DNA from MCF10AT cells with BstUI, i.e. it could be amplified and labeled with green (uncut) or red (uncut when the DNA is digested with BstUI). Our results indicated that BRCA1 and E-cadherin CpG islands were fully methylated even in the non-tumorigenic MCF10A cells, similarly to mitochondrial DNA (no BstUI sites) and ribosomal RNA (highly methylated). In the left panel CDH1, GSTP1, NOEY2 and p16 appear to be methylated, while ESR1 and p15 are unmethylated. In the right panel, BRCAI, HIC1, RBL and RIZ1 appear to be methylated while TIMP3 appears to be unmethylated. Data analysis of signal intensity from each probe was made after balancing the red/green signal from mitochondrial DNA to 1 as described in [5] verified these "calls". This array pattern did not differ between the MCF10 derived lines, i.e, during the establishment of the lesion-forming MCF10AT line from MCF10A or selection of more malignant derivatives of MCF10AT. However, although methylation of BRCA1 and E-cadherin "makes sense" in terms of breast tumor biology, the cloned regions from the CpG islands in both genes contained Alu repeats which have the potential to anneal with the PCR products amplified from Alu repeats elsewhere in the genome. This would give a strong "methylated" signal, since the Alu repeats are generally highly methylated. **Solution:** Remove the Alu repeat from the CpG island sequences spotted on the microarray. The PCR primers used to produce the E-cadherin and BRCA1 sequences are in the process of being redesigned.

2. *Analysis of DNA methylation of candidate genes by methylation specific PCR and sequencing of bisulfite treated DNA.* We have completed examination of CpG island methylation of four genes in DNA from MCF10A and the six tumor cell lines derived from it. Methylation of TIMP3 (tissue inhibitor of metalloproteins); WT1 (Wilm's tumor suppressor gene); NOEY2 (a member of the ras superfamily) and APC (Adenomatous Polyposis Coli) was examined using both methylation specific PCR and bisulfite sequencing. We have also examined GSTP1 and ESR1 by methylation specific PCR in MCF10A and MCF10AT (GSTP1 was methylated and ESR1 was not (data not shown and [6]) and are in the process of completing bisulfite sequencing and methylation specific PCR in DNA from the rest of the MCF10AT-derived lines. The same information was obtained from MCF7 cells to allow comparison with data published by others

With one exception (TIMP-3), the results obtained by CpG island microarray analysis are consistent with those we find by methylation specific PCR and bisulfite sequencing. Again, no significant change in the pattern of methylation of these genes between the different MCF10 derived lines was found using methylation specific Q-PCR of bisulfite treated DNA although differences between MCF10 derived lines and MCF7 cells were detected. (Figure 2)

Methylation specific Q-PCR of bisulfite treated DNA indicates that:

1) APC is unmethylated in all lines examined although there is a low level of random methylation in MCF7 DNA.
2) WT1 is fully methylated in all lines, but unmethylated in normal genomic DNA (Roche).
3) NOEY2 is fully methylated in all lines but not in normal human genomic DNA, where both methylated and unmethylated alleles are present (imprinted).
4) TIMP3 appears to be methylated on at least one allele in the MCF10A lines and normal genomic DNA, although a population of unmethylated alleles is also present. In contrast, TIMP3 is completely unmethylated in MCF7. In normal human genomic DNA, there appear to be both methylated and unmethylated alleles of TIMP3. This pattern is similar to what is observed in normal human genomic DNA for the imprinted NOEY2 gene.

The results of bisulfite sequencing of cloned PCR product of all 4 genes (APC, NOEY2, WT1 and TIMP3 are presented in Figure 3. By all three methods of analysis APC was unmethylated in all lines examined; NOEY2 and WT1 were methylated in all lines examined; TIMP3 was unmethylated in MCF7. Similarly, all data collected to date for ESR1 and GSTP1 methylation by bisulfite sequencing support the results from CpG island microarray and methylation specific PCR, i.e., GSTP1 is methylated, ESR1 is unmethylated. However, methylation specific PCR and bisulfite analysis indicated significant methylation of TIMP3 in all of the MCF10 lines, while the same CpG island region appeared to be unmethylated by microarray. The basis for this discrepancy is probably due to the presence of a BstUI (CpG) site within the 1481 bp long MseI genomic fragment that lies outside of the bisulfite sequenced area (588 bp long). While this site does not appear in GenBank sequences, we have found other "missing" BstUI sites due to sequencing errors in the original accessions. **Solution:** We are sequencing the entire region to determine where this BstUI site lies. This is underway.

3. *Expression of APC, TIMP3, NOEY2 and WT1 mRNA in MCF10A and MCF10A derived lines*
The results of analysis of mRNA levels by standard RT-PCR amplification (35 cycles) followed by gel electrophoresis and Southern blotting is presented in Figure 4. This semi-quantitative analysis of RT-PCR products (PCR of various mRNAs compared to GAPDH as reference gene) suggests that TIMP3 and APC are expressed at relatively high levels in all cell lines and in normal breast tissue, that WT1 is expressed at a barely detectable level in all of the cell lines (but not in the normal breast tissue) and that NOEY2 is highly expressed in two of the MCF10CA lines and normal breast but is expressed at relatively low levels in MCF10A,10AT and two different MCF10CA lines.

To confirm and extend these observations, quantitative real time RT Q-PCR was carried out using standard protocols [7]. The data was analyzed using GAPDH as the reference gene and MCF10, the non-tumorigenic precursor of the other MCF10 lines as the "calibrator sample" for determining the relative level of expression of each individual gene. The data (below) are consistent with the standard RT-PCR evaluation. APC levels are 2-3 fold lower in the highly malignant CA and DCIS lines than in MCF10A and AT. In contrast, NOEY 2 is expressed at 10-100 fold higher levels in these malignant line than in MCF10A, while MCF10AT actually expresses ~5 fold less NOEY2 than MCF10A. Little difference was observed in the low level of WT1 expression in the MCF10A derived lines, but all these lines expressed at least 6 fold less WT1 mRNA that MCF-7. TIMP3 expression is readily detected in all lines (Fig. 4) but was particularly high in MCFCA1h cells which form metastatic tumors.

EXPRESSION OF FOUR TUMOR SUPPRESSOR GENES DETERMINED BY REAL TIME Q-PCR

Cell Line	**MCF10A**	**MCF10AT**	**CA1a**	**CA1d**	**CA1h**	**DCIS.com**	**MCF-7**
Gene							
APC	1	0.741	0.549	0.397*	0.334*	0.354*	1.664
NOEY2	1	0.235*	15.269**,***	10.321**	32.354***	96.955#	0.537^{+},*
WT1	1	0.426^{+}	1.663^{++}	0.524^{+}	0.881^{++}	1.036^{++}	6.652*
TIMP3	1	1.289	0.147**	2.763***	11.441*	6.060*	0.954

Values calculated by the $2^{-\Delta\Delta CT}$ method [1] . PCR conditions for all amplifications were properly optimized and efficiency was close to 1. Thus, each number represents the fold difference in mRNA level relative to the base value in MCF10A. All points are derived from the mean value of three determinations.
*, ** and *** indicate homogenous subgroups that are similar to one another and different from MCF10A and the other subgroups with a >95% probability. + and ++ indicate homogenous subgroups similar to MCF10A but different from each other with >95% probability. Absence of an * indicates the values are the same as MCF 10A with a >95% probability. # indicates unique value

Although much more data will need to be analyzed, we can already come to the conclusion that the conversion of non-tumorigenic MCF10A cell line to a line capable of forming lesions characteristic of slow growing pre-neoplastic lesions does not involve methylation mediated silencing of APC (compare 10A/10AT). However, three of the four highly tumorigenic lines do express significantly lower levels of this mRNA than 10A without increased methylation. WT1, which is expressed at low levels in all cells, is also methylated in all of these cells. NOEY2

mRNA expression is higher than that in MCF10A or AT in all four of the highly tumorigenic lines although it is not linked to the capacity to form tumors (compare 10A/10AT with MCF7) or to metastasize (compare metastatic CA1h with CA1d or DCIS). Interestingly, its expression levels are not linked to methylation changes in the CpG island in the promoter region that was studied here (i.e., this CpG island is fully methylated in both expressing and non-expressing cells). However, a second CpG island region associated with methylation mediated gene silencing has been reported in this gene and we will evaluate its methylation as well.

At this point is difficult to assess the results we obtained with TIMP3, a gene whose mRNA is highly expressed in all lines, since there is little concordance between results obtained with the CpG island microarray (no methylation) and that obtained with bisulfite sequencing (heavily methylated in all cell lines except MCF7 where it is completely unmethylated). However, the possibility still exists that the region containing the BstUI site that leads to the results found in the microarray will prove to be in a more critical regulatory region of the TIMP3 CpG island than the area examined in our current studies.

It should be emphasized that all of the four CpG islands we have studied in detail have been implicated by other groups as being sites for aberrant methylation in breast cell lines or tumors [8-13]. We have either used the same primers/probes or new primers in the same region that were developed to improve the efficiency of PCR (Tables 1-3) Thus, while we can clearly concluded that there is little or/no change in the methylation of the genes regions we have studied during the derivation of the MCF10A model system for progression, there are still significant differences in the expression levels of the mRNAs encoded by the genes adjacent to these CpG islands.

The lessons learned from our mini-microarray analysis with arrays based on the sequence of MseI fragments that would be present in the CpG island library have proven instructive with regard to the limitations of these microarrays. Thus, we will continue to refine our mini-microarrays of known relevant genes to ensure that they are not missed in our unselected CpG island arrays and will initiate our search for genes that may be related to progression using our 40,000 CpG island library. We anticipate completing these studies for the cultured cells within the next 6 mos. and to have completed initial analysis of the tumor samples with in the next 12 mos.

KEY RESEARCH ACCOMPLISHMENTS

- Optimized all of the techniques required for isolation of and analysis of DNA and mRNA needed for studies of CpG island methylation by methylation specific PCR and CpG island array from cultured cells.
- Completed analysis of the methylation patterns of four known CpG islands (NOEY2, TIMP3, APC and WT1) in 6 cell lines representative of different stages in the MCF10A model of breast cancer progression (10A/AT/CA1a/CA1d/CA1h/DCIS.COM). Partial completion of analysis of GSTP1 and ESR1 (10A/AT)
- Collected sufficient tumor mass from highly tumorigenic MCF10AT derivative lines for analysis of methylation and expression of key tumor suppressor genes by methylation specific PCR.and Q-RT-PCR,
- Initiated isolation of areas with the morphology of carcinoma in situ from DCIS.COM and CA1a, CA1d and CA1h xenografts by laser capture.

III. REPORTABLE OUTCOMES

None. Ms. on methylation of TIMP3, NOEY2, APC and WT1 in preparation.

IV. CONCLUSIONS

Within the limited range of genes examined to date, it is clear that valuable information as to the methylation status and expression of cancer related genes can be gained through use of microarray technology. Our experience with mini-microarrays of known genes points out the importance of constructing such arrays for actual diagnosis of methylation changes and of validating the concordance between array data and data obtained by analysis of bisulfite treated DNA. We plan to refine and expand this mini array during the next year adding genes whose silencing has been detected by gene expression microarray. This approach will enhance our progress toward the goal of discovery of methylation changes critical to the early stages of breast cancer progression.

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Table 1 MSP primer sequences[a], annealing temperature and products size

Gene	Forward primer(5'→3')	Reverse primer(3' →5')	Annealing temp(℃)	Product size(bp)
APC[c]	M: TATTGCGGAGTGCGGGTC [b]	M: TCGACGAACTCCCGACGA	61	98
	U: GTGTTTTATTGTGGAGTGTGGGTT	U: CCAATCAACAAACTCCCAACAA	55	108
NOEY2	M: GTATTCGTCGTTAGGCGTTT	M: CGAAACTTTCGTTATTCTTCGC	57	300
	U: AATGTTTGGTAGGTGGTGGTG	U: CCTCACCAAACCCTCACAAAA	61	225
TIMP3	M:TACGAGGGTTTCGTTTCGAGGA	M: CCGCCTCGAACTATTAAAACCCG-	59	231
	U:GGGGTATGAGGGTTTTGTTTTG	U: AACCACATTACCTCATCAACCC	57	192
WT1	M: TTTGGGTTAAGTTAGGCGTCGTCG	M: ACACTACTCCTCGTACGACTCCG	63	353
	U: TTTGGGTTAAGTTAGGTGTTGTTG	U: ACACTACTCCTCATACAACTCCA	59	353

[a]References for primer sequences: APC from Esteller M, et al. Cancer Research, 2000, 60: 4366-4371; WT1 from Loeb DM, et al. Cancer Research, 2001, 61: 921-925.
[b] M, methylated-specific primers; U, unmethylated-specific primers.
[c]Accession number: U02509 and AC136500 for APC, AF202543 for NOEY2, AF 01361 for TIMP3, AL049692 for WT1.

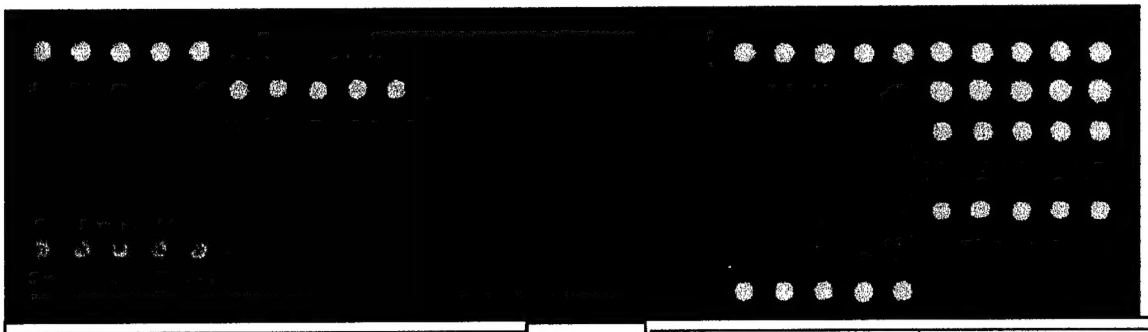
Table 2 Primer sequences for bisulfite sequencing[a]

Genes	Accession number	Sequences	Annealing temperature(℃)	Product size(bp)
APC	U02509 AC136500	Forward: 5-TTTTGTTTGTTGGGGATTGGGGT-3 Reverse: 5-CAATAACACCCTAACRAACTACACCA-3	59	261
NOEY2	AF202543	Forward: 5-GGYGGTGGTGYGTAGTTTTTAAT-3 Reverse: 5-TTCRTTATTCTTCRCCTCCCACT-3	58	316
TIMP3	AF01361	Forward: 5-GGGYGTGGGGTTAGGGYGTAGA-3 Reverse: 5-AAACTACTACTCRCCTCTCCAAAATTACC-3	61	588
WT1	AL049692	Forward: 5-GTAGTTAGAGTAGTAGGGAGTT-3, Reverse: 5-CTCCRACTATACCAATAAACTAACC-3	57	443

[a]Degenerate primer, Y=C/T, R=G/C

Table 3 Primer sequences for reverse transcriptional PCR and real time PCR

Genes	Accession number	Sequences	Exons	Annealing temperature(℃)	Product size(bp)
GAPDH	J04038	F: 5-CCTGACCTGCCGTCTAGAAAA-3 R: 5-CCCTGTTGCTGTAGCCAAATT-3	8 9	60	239BP
APC	M74088	F: 5-GGACTGTGAAATGTACGGGCTT-3 R: 5-GGACACATTCCGTAATATCCCA-3	11 14	60	478bp
NOEY2	U96750	F: 5-CCGAGCAGCGCATTTGTCTT-3 R: 5-CGATGGGGAACTTATGCAGGTT-3	1 2	60	558bp
TIMP	NM000362	F: 5-GCGTCTATGATGGCAAGATGTA-3 R: 5-CAGGTTCCTCATTCTTTCTGGCA-3	4 5	60	583bp
WT1	AL049692	F: 5-CCAGGGCATGTGTATGTGTC-3 R: 5-GATCCTGGACCATCCCCTAT-3	3' UTR 3' UTR	59	519bp



MITOCHONDRIA	APC
P1A7	CDH1
P1B3	p16
P1F4	p15
P1H5	ESR1
P1H12	GSTP1
NOEY2	

MITOCHONDRIA	18s RNA (1)
HIC1	18s RNA (2)
MGMT	MITOCHONDRIA
MLH1	RIZ1
MYOD1	BRCA1
RBL	TIMP3
MITOCHONDRIA	

Figure 1. CpG island "mini-microarray" with cloned CpG islands from the indicated genes was probed with a mixture fluorescent tagged amplicons of MseI cleaved DNA (Cy3-green) and MseI/BstUI cleaved DNA (Cy5-red) from MCF10AT cells. P* designation is for CpG island clones used as a check for digestion and balancing of fluorescence from the probes. The ratio of "red/green" emission from P1H12, a reference sequence with no BstUI cleavage sites is set to 1.00. Green color indicates that all DNA with BstUI sites was cleaved (unmethylated) while yellow color indicates either the lack of BstUI sites or methylation of BstUI sites. See text for interpretation.

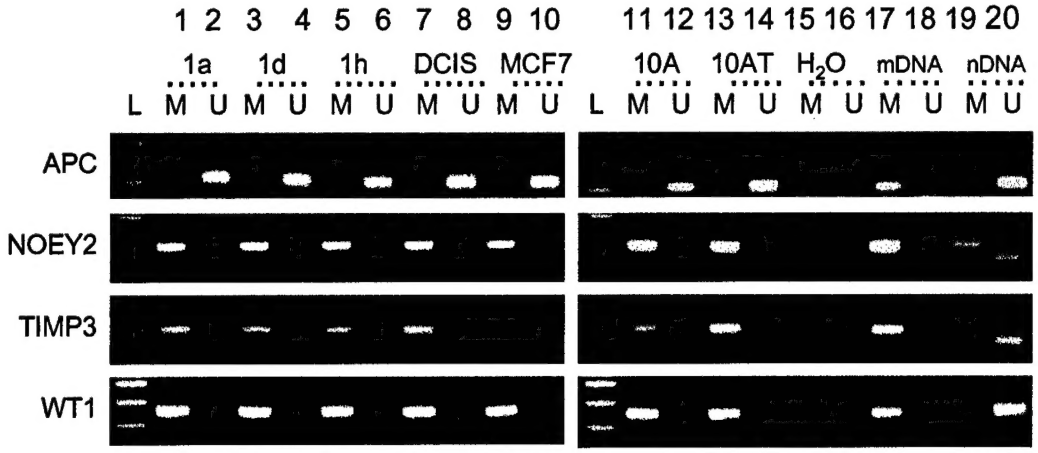


Figure 2. Methylation specific PCR of CpG island regions in the indicated tumor suppressor genes. M indicates primer specific for bisulfite treated methylated DNA; U indicates primers specific for bisulfite treated unmethylated DNA. Controls: Lane 16, no template; SssI methylated DNA; normal human genomic DNA. Lanes 1-6, tumorigenic MCF10CA lines (MCF10 CA1h is also metastatic); Lane 7,8 MCF10 DCIS.com; Lanes 9,10, MCF7 cells, Lanes 11,12, MCF10A-parental line; Lanes 13,14; MCF10AT, forms slow growing lesions with areas of DCIS and locally invasive cancer. Note that APC is unmethylated in all lines, WT1 and NOEY2 are fully methylated in all lines but not in normal human genomic DNA and TIMP3 appears to be methylated on at least one allele in MCF10A lines and normal genomic DNA but completely unmethylated in MCF7 cells.

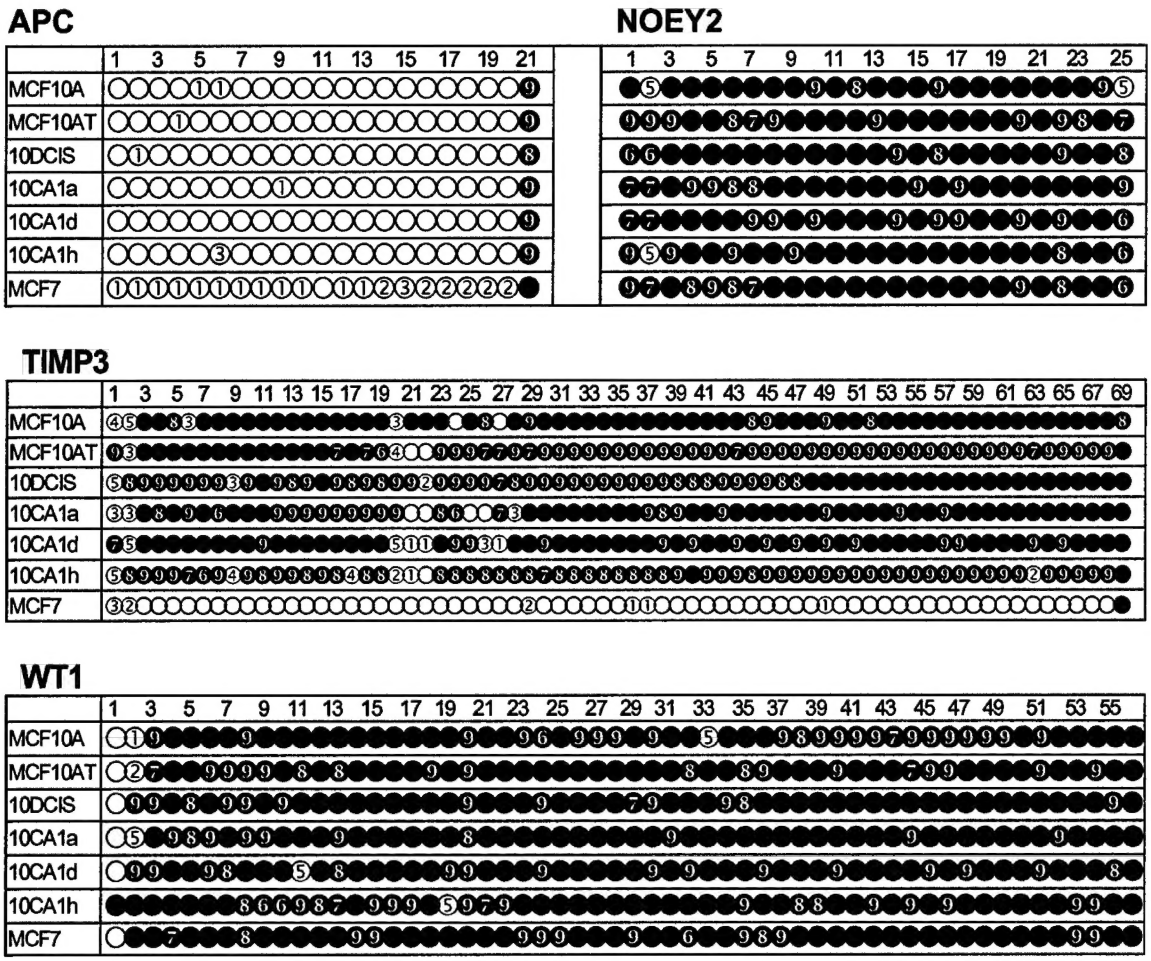


Figure 3. Bisulfite sequencing of APC, NOEY2, TIMP3 AND WT1 genes in MCF7, MCF10A and lines derived there from. Bisulfite treated genomic DNA from each line was amplified, cloned and sequenced. Ten to twelve clones from each PCR product were analyzed to obtain the frequency of methylation at all CpG sites within the amplified region. Results are expressed as percent of clones methylated at site. Filled circles were methylated in more than 50% of clones examined; empty circles were methylated in fewer than 50% of clones examined. Numbers within circles represent actual frequency of methylation at a site. In absence of a number, empty circles indicate no methylation and full circles 100% methylation. In all cases where multiple scores of 9 are recorded, one of the clones was unmethylated at all of the indicated sites.

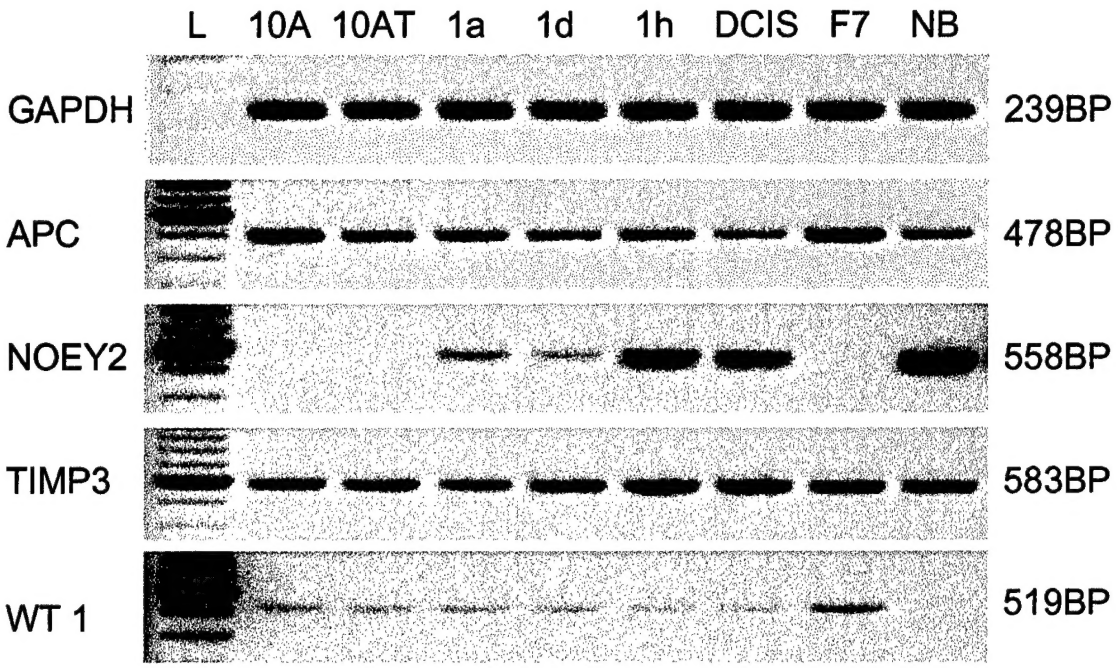


Figure 4. RT-PCR analysis of APC, NOEY2, TIMP3 AND WT1 gene expression using GAPDH as an internal control. NB is commercially RNA available normal human breast tissue (Ambion, Top Choice total RNA). Other designations as in Figure 2. cDNA was amplified for 35 cycles. All samples are PCR product from a 5 µl reaction mixture with the exception of TIMP3. Only 2 µl of this reaction was loaded to avoid overexposure due to high concentration of labeled PCR product.